# New non-woven polyurethane-based biomaterials for the cultivation of hepatocytes: expression of differentiated functions

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A new non-woven polyetherurethane support suitable to host cultured hepatocytes has been developed. Prior to its use in bioreactors and artificial liver devices, the biocompatibility of this new material was investigated. The experiments have shown that the survival and functionality of hepatocytes entrapped in the non-woven polymer were longer than that of monolayer cultured hepatocytes, under serum-free culture conditions. Hepatic specific metabolic functions, namely, synthesis of urea and synthesis and secretion of plasma proteins, were well maintained by hepatocytes entrapped in non-woven polyetherurethane sheets. Cells also retained the expression of biotransformation activities of 7-ethoxycoumarin-*O*-deethylase as well as CYP2A1, CYP2B1 and CYP3A1. The results presented in this paper point to non-woven polyetherurethane sheets as a suitable biocompatible support for functional, three-dimensional hepatocyte cultures.


## 1. Introduction

Three-dimensional, high density functional cultures of hepatocytes are of interest both for the design of bioreactors for *in vitro* investigation of hepatic metabolism of xenobiotics and for bioartificial liver support systems for temporary, extracorporeal therapy for patients with liver failure bridging to liver transplantation. Different cell supports have led to the development of bioartificial liver devices that allow high cell densities and close contact of the cells to the patient's blood. Cells are cultivated inside capillary membranes embedded in a collagen matrix [1, 2], microbeads entrapped in capillary systems [3, 4], three-dimensional woven structures of capillaries [5], polyamide hollow fibers, cellulose and polypropylene [2, 5, 6], reticulated polyurethane [7] and non-woven polyester material [8, 9].

The aim of the present study was to explore the suitability of a new polyurethane-based non-woven three-dimensional matrix to host hepatocytes in a way that they express the differentiated adult phenotype. Several specific hepatic functions (ureogenesis, albumin synthesis and biotransformation activities) were examined and compared with those expressed by hepatocytes cultured on tissue culture polystyrene (TCP). The results presented in this paper show that non-woven polyetherurethane sheets are a suitable biocompatible support for functional, three-dimensional hepatocyte cultures.

## 2. Material and methods

### 2.1. Preparation of the non-woven biomaterial

The non-woven biomaterials were made of polyetherurethane (Tecoflex® EG 85A/Thermedics Inc., Woburn, USA) by a fiber extrusion technology. In this process, melted polymer is extruded through capillaries forming microfibers. The fibers are laid down on a conveyor belt to a non-woven structure [10]. No additives, solvents or further processing were used. The non-woven tissue was cut into round circles (15 cm diameter), laid down inside 24-well culture plates, and fixed in with 0.1 ml of collagen solution (0.1 mg $ml^{-1}$ collagen type I in Dulbecco's modified Eagle's culture medium). After drying the plates were sterilized by $\gamma$-irradiation.

### 2.2. Scanning electron microscopy of non-woven polyetherurethane

The structure of the non-woven polyetherurethane was analyzed in a scanning electron microscope (SEM, Zeiss DSM 950). Small pieces of the material were coated in a sputter coater with a layer of gold/palladium (approximately 15 nm). Filament diameters and pore dimensions were determined in the SEM.

### 2.3. Isolation and culture of hepatocytes

Hepatocytes were isolated from Sprague–Dawley male rats (180–250 g) by reverse perfusion of the liver with collagenase, as previously described [11], and were resuspended in Ham F-12/Leibovitz-15 medium supplemented with 2% newborn calf serum, 0.2% bovine serum albumin (BSA), $10^{-8}$ M insulin and antibiotics [11, 12]. Finally, hepatocytes were seeded either on tissue culture dishes (TCP) or on top of the non-woven material coated with fibronectin ($3.5\,\mu g\,cm^{-2}$) at a final density of $80 \times 10^3$ viable cells/cm$^2$. Medium was renewed 1 h after cell plating. Twenty-four hours later, cultures were shifted to serum-free, hormone-supplemented medium ($10^{-8}$ M dexamethasone and insulin).

### 2.4. Cell viability assay (XTT assay)

Cell viability was assessed by the XTT test, which is based on the bioreduction of 2,3bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner sodium salt (XTT) to a water-soluble formazan by the mitochondrial succinyl dehydrogenase, as described elsewhere [13].

### 2.5. Hepatic metabolic function determination

Urea and albumin synthesis rates were evaluated by measuring either the urea or albumin secreted in 2 h, in aliquots of culture medium. The maximal ureogenesis rate was determined after incubating the hepatocytes with 3 mM ammonium chloride and the albumin secretion rate was determined by enzyme-linked immunosorbent assay (ELISA), as described in detail elsewhere [14]. DNA was fluorimetrically quantified using the fluorochrome Hoechst 33258 [15].

### 2.6. Measurement of drug metabolizing activities

7-Ethoxycoumarin-*O*-deethylase (ECOD) activity was directly assayed fluorimetrically in intact cells incubated with medium containing 150 μM 7-ethoxycoumarin as described in detail previously [16]. Testosterone hydroxylation was measured in intact hepatocytes incubated for 30 min with 250 μM testosterone. Metabolites (CYP1A1, 2B1, 3A1 and 2C11) were extracted from culture medium with ethylacetate and analyzed by high pressure liquid chromatography (HPLC) as described [16].

## 3. Results

### 3.1. Physical properties and ultrastructure of the non-woven material

Adjustment of the processing parameters made it possible to conform most of the characteristics of the non-woven material which are crucial for a three-dimensional cell culture system. Not only the thickness of the non-woven material and its filaments but also the dimensions of the pores and channels inside could be adjusted in a wide range. As standard material for this study, 2 mm sheets had been prepared. In Table I the characteristics of the non-woven material are summarized. The pore volume describes the ratio of solid polymer to ''air'' inside the non-woven material. The pores were interconnective and their dimensions varied in a wide range. The non-woven process had been adjusted to resulting pore dimensions which permitted the penetration of the cells into the structure but prohibited the flow of the cells through the material. The structure had been selected in screening experiments using long term cellular functionality and survival as criteria. It can be observed in the micrograph shown in Fig. 1.

### 3.2. Survival and hepatic metabolic functions of hepatocytes cultured in the non-woven biomaterial and on tissue culture polystyrene (TCP)

Once hepatocytes had been allowed to enter the non-woven polyetherurethane, cell survival was periodically determined by measuring DNA entrapped in the polymer. In parallel, cellular viability was monitored by the XTT test. As shown in Fig. 2a, the number of cells in the non-woven polymer (expressed as DNA) was larger, and they survived longer than hepatocytes

TABLE I Properties of the non-woven polyetherurethane

| Properties | |
|---|---|
| Thickness of the non-woven sheet | 2 mm |
| Fiber thickness | 20–60 μm |
| Pore volume | 70% |
| Pore diameter | 10–200 μm |
| Pore depth | up to 500 μm |

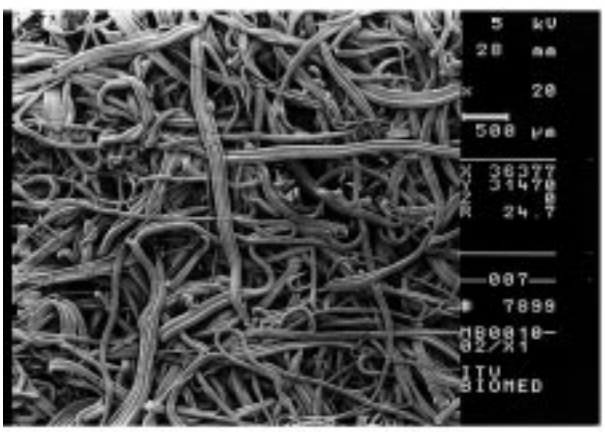


*Figure 1* Structure of the non-woven material by scanning electron microscopy.

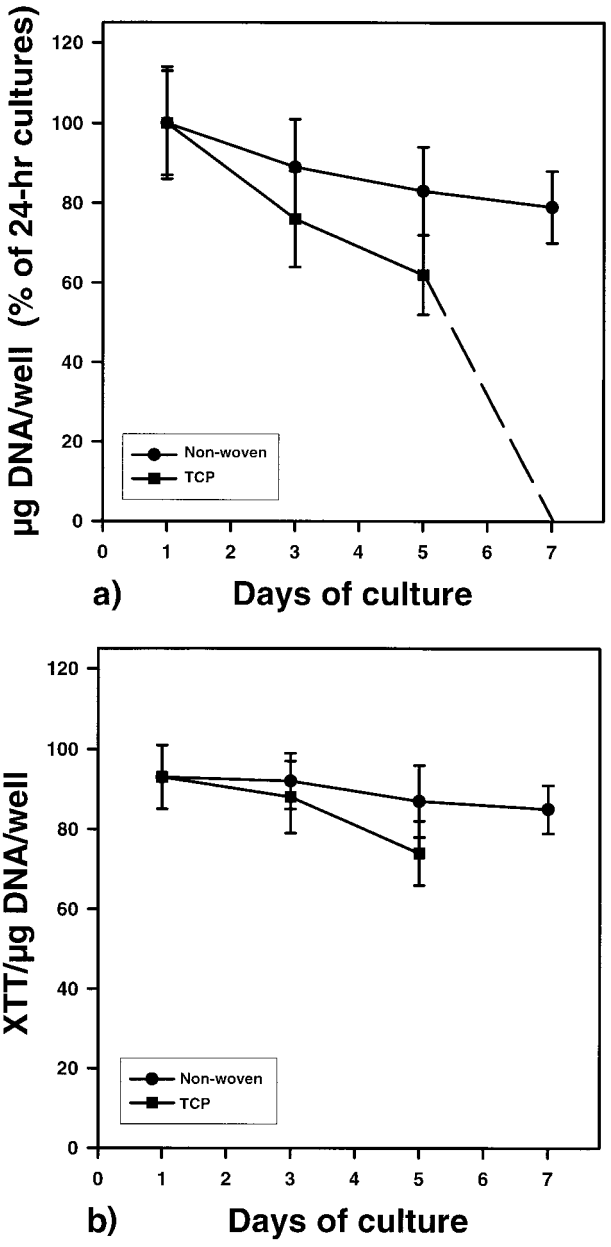


*Figure 2* Survival of cultured hepatocytes. (a) Cell survival is expressed as a percentage of DNA with respect to 24-h cultures. (b) Viability of hepatocytes entrapped on the polymer was monitored by the XTT test and the results show the XTT/DNA ratio of either non-woven polyetherurethane-entrapped hepatocytes (dark symbols) or hepatocytes cultured on tissue culture polystyrene (TCP, clear symbols). Data are the mean ± SD of three different cultures.

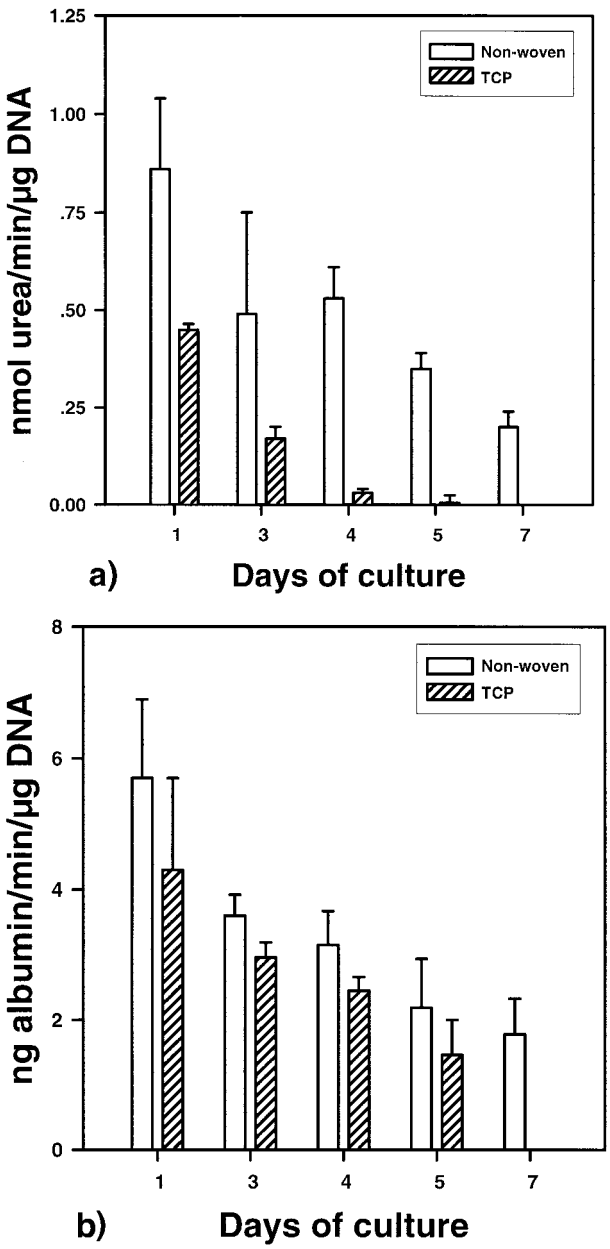


*Figure 3* Hepatic metabolic functions in cultured hepatocytes. (a) The urea synthesis rate was determined after loading hepatocytes with 3 mM ammonia. (b) Albumin synthesis was measured in polyurethane cultures (open bars), as well as in tissue culture polystyrene (TCP, shaded bars). Data represent the mean ± SD of three different cultures.

cultured on TCP. That hepatocytes retained in the non-woven sheet are alive is shown by the results depicted in Fig. 2b. Moreover, the XTT/DNA ratio remained stable in polymer-entrapped cells, while in TCP cultured cells it clearly decreased.

To assess the functionality of polyetherurethane-entrapped cells, representative hepatocyte metabolic functions were investigated in both culture conditions. To measure the ureogenic rate of the hepatocytes cells were incubated in culture medium containing 3 mM ammonium chloride. In hepatocytes entrapped in the non-woven material, the ureogenic rate remained fairly stable for 4 days in culture and close to 24-h values ($0.86 \pm 0.18$ nmol min$^{-1}$ × µg DNA). Conversely, rapid decreasing rates which were undetectable on the fourth to fifth day of culture were observed in monolayer hepatocytes cultured on TCP (Fig. 3a).

The synthesis of albumin steadily decreased in both types of culture conditions. However, the rates measured with hepatocytes entrapped in the non-woven polymer were slightly higher and were maintained longer than with TCP-cultured hepatocytes, (Fig. 3b).

### 3.3. Biotransformation capability of hepatocytes cultured in non-woven polyetherurethane.

7-Ethoxycoumarin-*O*-deethylase activity (ECOD), which is associated with several cytochrome P450 isoenzymes, decreased in both culture models with time. The major difference, however, was the fact that polymer cultured hepatocytes still expressed biotransformation activities after 7 days (ca. 40% of initial values), while in TCP cultures this activity was undetectable (Fig. 4).

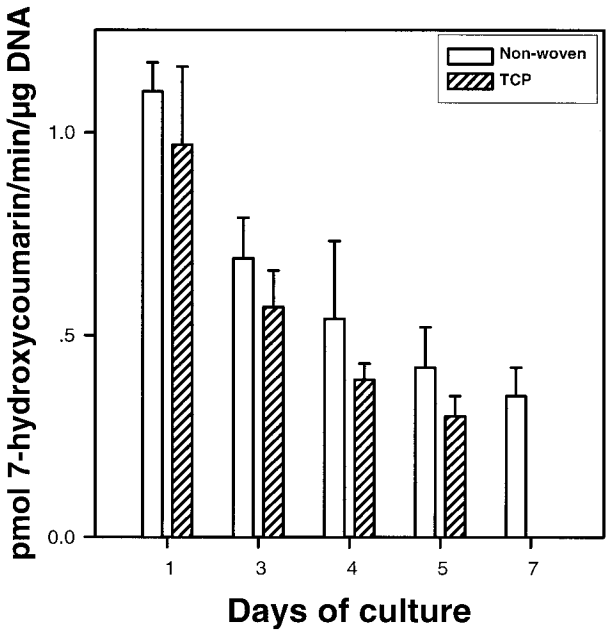


*Figure 4* Expression of drug-metabolizing activities in culture. 7-Ethoxycoumarin *O*-deethylase (ECOD) was determined in polymer cultured hepatocytes (open bars) and in tissue culture polystyrene (TCP, shaded bars) along time in culture. Data represent the mean ± SD of three different cultures.

Testosterone is regio- and stereoselectively metabolized by human CYPs to several hydroxylated metabolites [17]. The rate of formation of the different testosterone hydroxylated metabolites was also monitored in culture medium to quantify specific CYP activities. As shown in Table II, all the activities assayed, namely 16-α hydroxylation (catalyzed by CYP2B1), 6β- and 15β-hydroxylation (catalyzed by CYP3A1) and 7α-hydroxylation of testosterone (catalyzed by CYP2A1) were clearly measurable after 7 days in polyetherurethane-entrapped hepatocytes. In TCP-cultured cells the hydroxylation rates were significantly lower by the fourth day of culture, and fell below the detection limit by the fifth day of culture.

## 4. Discussion

Functional long-term survival of hepatocytes is a critical issue both in the design of hybrid artificial liver and for liver bioreactors [17, 18]. In this context, it appears that the characteristics of the materials used as culture substratum are of key importance, not only to allocate high density of cells but, even more important, to allow cells to recreate a three-dimensional structure that mimics the situation of cells in the liver, and avoid the de-differentiation observed in monolayer cultures [19–21]. Different strategies have been developed for hepatocyte immobilization in three-dimensional matrices to maintain cell viability and functionality over long periods of time [17]. The suitability of collagen [22, 23], extracellular matrix components [24], gelatin sponges [25], hydrogels [26], and alginate gels [18] has been explored with variable degrees of success. However, their use in liver bioreactors or artificial liver devices is very limited because of their low mechanical resistance.

The use of non-woven synthetic fibers as three-dimensional cell carrier had also been considered for several applications [8, 27] with varying success. One of the most important criteria in selecting a material had been its availability but the materials had not been modified for the specific application. The non-woven material that has been used for the experiments here has been selected by a great number of screening experiments: polyetherurethane has been chosen due to its good biocompatibility and the encouraging experience the authors have using it with a great variety of cell types. The crucial property for the functionality of the hepatocytes appeared to be the pore geometry of the material. The non-woven process was best suited to obtain pores up to 200 µm which resulted in a three-dimensionally well-distributed culture of small aggregates of hepatocytes.

In view of these results we explored the suitability of non-woven polyetherurethane to support functional cultures of hepatocytes for use in liver bioreactors. A primary requirement was not only cell survival but also the long-term preservation of *in vivo* metabolic functions. The polyurethane-based polymer showed a high degree of biocompatibility. The number of cells entrapped in the non-woven material was large and remained fairly stable after several days in culture, but, most important, when the viability of cells was checked, it became evident that most of cells retained in the polymer were alive (constant XTT/DNA ratio). This contrasts with the situation observed in monolayer cultures, where both the number of cells and viability decreased drastically after 4–5 days in culture.

The polyetherurethane-based culture substratum showed interesting properties in relation to the expression of differentiated functions by hepatocytes. Two of them were examined: ureogenesis and plasma protein synthesis. Cells were able to produce urea from ammonia

TABLE II Testosterone hydroxylation in hepatocytes cultured in non-woven polyetherurethane and on tissue culture polystyrene (TCP)

| Days | | Testosterone metabolites (pmol/h/µg DNA) | | | |
|---|---|---|---|---|---|
| | | CYP3A1[a] | CYP2A1 | CYP3A1[b] | CYP2B1 |
| 5 | TCP | ud | ud | 1.84 | ud |
| 5 | Non-woven | 2.95 | 12.10 | 29.30 | 7.00 |
| 7 | Non-woven | 1.70 | 6.70 | 11.90 | 5.50 |

Hepatocytes were incubated with 250 µM testosterone for 1 h. Metabolites released to the medium were extracted and analyzed by HPLC.
ud: undetectable.
[a]15β-hydroxytestosterone.
[b]6β hydroxytestosterone.

at a rate close to that of 24 h cultured cells, and retained the ability to synthesize albumin. Both facts show that the non-woven material can host cells in a way that they retain their metabolic competence.

For a hepatocyte to be used in a bioreactor it is critical that it expresses biotransformation activities at a level comparable to that *in vivo*. For this reason, different CYP activities were examined in the polymer-entrapped cells after seven days in culture. The 7-ethoxycoumarin-*O*-deethylase activity (ECOD) is catalyzed by CYP1A1, 2A1, 2B1 and 2B2 in rat hepatocytes [28]. As the results in Fig. 4 summarize, non-woven entrapped cells showed ECOD activities which, after 7 days in culture, were still approximately 50% of the day 1 values. Individual quantification of CYP2A1, CYP2B1 and CYP3A1 consistently showed that the three isoforms were expressed by polyetherurethane entrapped cells, while these activities were undetectable in conventional cultures.

The maintenance of a differentiated cell status, as it is found in the adult liver, may require not only soluble signals delivered by appropriate supplementation of culture media, but also by cell-to-cell and cell–matrix interactions. Both regulate the transcription of hepatic genes [29, 30], but the latter are more difficult to mimic in conventionally cultured cells. Scanning electron microscopic analysis indicated that the fibers of the non-woven material, because of its size and distribution, could provide a framework to allow three-dimensional cell-to-cell contact among entrapped hepatocytes. Subsequent reorganization of cells into aggregates within polymer spaces could also contribute to the long-term expression of the adult phenotype of hepatocytes as shown in our experiments [3, 8].

The results presented in this paper suggest that non-woven polyetherurethane sheets are a suitable biocompatible support for three-dimensional, functional hepatocyte cultures.

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